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Enhancement of the Thermostability of a Fibrinolytic Enzyme from *Bacillus amyloliquefaciens* CH51

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AprE51 from *Bacillus amyloliquefaciens* CH51 is a 27 kDa subtilisin-like protease with fibrinolytic activity. AprE51-6 showing increased catalytic activity was produced previously. To enhance the thermostability of AprE51-6, 2 residues, Gly-166 and Asn-218 based on *B. subtilis* subtilisin E were mutated by site-directed mutagenesis. The results of the mutational analysis showed that substitution of arginine for Gly-166 (AprE51-7) increased the fibrinolytic activity 1.8-fold. An N218S mutant (AprE51-8) also increased the fibrinolytic activity up to 4.5-fold in a fibrin plate assay. Purified AprE51-7 and AprE51-8 mutants had a 1.9- and a 2.5-fold higher k_{cat}, respectively, and a 2.1–1.9-fold lower K_m, respectively. This resulted in a 3.8- and a 4.7-fold increase in catalytic efficiency (k_{cat}/K_m), respectively, relative to that of wild-type AprE51. AprE51-8 had a broader pH range than AprE51-6 and nattokinase, especially at an alkaline pH value. In addition, AprE51-8 showed higher thermostability than AprE51-6 at 60°C. The half-lives of AprE51-7 and AprE51-8 at 50°C were 21.5 and 27.3 min, respectively, which are 2.0 and 2.6 times longer, respectively, than that of the wild-type AprE51.

Key words : *Bacillus amyloliquefaciens*, *Cheonggukjang*, fibrinolytic enzyme, *in vitro* mutagenesis, thermostability

Introduction

Fibrin, the primary protein component of a blood clot over a wound site, is formed from fibrinogen by thrombin and lysed by plasmin activated from plasminogen. Under an unbalanced situation between fibrin formation and dissolution of blood clot, it may cause bleeding or formation of an inappropriate clot. When accumulated in the blood vessels, fibrin can interfere with blood flow and cause thrombosis leading to cardiovascular diseases [11]. Cardiovascular diseases, such as acute myocardial infarction and ischemic heart disease, imperil the human lives and health in modern life [11]. Thus, thrombolytic agents to lyse thrombus in blood vessels have been extensively investigated [2, 5].

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Recently, the fibrinolytic enzymes produced *Bacillus* sp. from Asian fermented foods, such as natto, douchi, doenjang, jeotgal, and cheonggukjang, have attracted interests as safer thrombolytic agents [4, 9, 17, 20]. Nattokinase (formerly designated subtilisin NAT, E.C. 3.4.21.62), which means 'enzyme in natto', is a potent fibrinolytic enzyme from *Bacillus subtilis* and was first found in the traditional Japanese soybean food natto [19, 20]. In addition, it also enhances the body's production of both plasmin and other clot-dissolving agents, including urokinase [19, 20]. However, the activity of nattokinase is sensitive to temperature and pH of its environment [20]. Thus, most studies of nattokinase have currently focused on enhancement of temperature, oxidation, and pH stability as well as fibrinolytic activity of enzyme by mutagenesis or encapsulation using various materials [3, 8, 23]. The thermal stability of fibrinolytic enzyme is also an important character for a product formulation and enzyme preservation in pharmaceutical industry [15].

B. amyloliquefaciens CH51 isolated from cheonggukjang, a traditional Korean fermented soy-bean food, showed higher fibrinolytic activity [12]. Previously, among fibrinolytic enzyme secreted by *B. amyloliquefaciens* CH51, the properties of AprE51, which is a 27 kDa subtilisin-like protease with fibrinolytic activity, were examined. Its structural gene,

aprE51, was previously cloned and engineered to improve its fibrinolytic activity by *in vitro* mutagenesis [12, 13]. AprE51-6 (S101W/G169A/V192A) was confirmed to have strong fibrinolytic activity than the wild-type AprE51 [13].

In this paper, we attempted to improve the thermostability of AprE51 by site directed mutagenesis. The fibrinolytic properties of AprE51 variants as potential thrombolytic agents in comparison with AprE51, AprE51-6, and nattokinase are presented.

Materials and Methods

Materials

Fibrinogen (from human bovine plasma), thrombin (from human plasma), and plasmin were purchased from Sigma-Aldrich. (St. Louis, MO, USA). The chromogenic substrate *N*-Succ-Ala-Ala-Pro-Phe-*p*NA (Succ: succinyl; *p*NA: *p*-nitroaniline) was also purchased from Sigma-Aldrich. Restriction endonucleases were purchased from Beamsbio (Sungnam, Korea). PCR Premix for PCR amplification was purchased from Genetbio (Daejeon, Korea). *Taq* polymerase, *Pfu* polymerase, and deoxynucleotide triphosphates (dNTPs) for site-directed mutagenesis were purchased from Takara (Shiga, Japan). A purification kit for PCR products and DNA restriction fragments and the QIAquick Gel Extraction kit were obtained from QIAGEN (Hilden, Germany). Bradford reagent kit and protein standard markers were purchased from BioRad (Richmond, CA) and Elpis-biotech (Daejeon, Korea). Enzymes were purified using HiTrap Q HP and HiTrap Phenyl HP, purchased from Pharmacia (Amersham Biosciences, Sweden). All other chemicals used were of analytical grade.

Bacterial strains and culture conditions

Escherichia coli DH5α was used for transformation and DNA manipulation. *B. subtilis* strain ISW1214 (Takara, Shiga, Japan) was used as a host for the expression of AprE51 mutants. Cells containing pHY51 derivatives were cultivated in LB supplemented with 100 μg/ml ampicillin or 10 μg/ml tetracycline.

Construction and expression of *aprE51* variants

The *aprE51-6* gene was previously constructed from *aprE51* gene of *B. amyloliquefaciens* CH51 and was cloned into pHY300PLK to generate pHY51 [13]. The *aprE51-7* and *aprE51-8* gene were constructed from *aprE51-6* gene by site-directed mutagenesis using the overlap extension method [7, 13]. Oligonucleotides for site-directed mutagenesis were synthesized by Genotech (Daejeon, Korea) and were listed in Table 1.

The wild-type and AprE51 mutants were expressed in the protease-deficient host *B. subtilis* ISW1214. Preparation and electroporation of competent *Bacillus* cells were carried out as previously described [13]. The expression and purification of AprE51-7 and AprE51-8 were performed as previously described [10, 13, 25].

Enzyme assay

Fibrinolytic activity was determined using the fibrin plate method [1]. The fibrin plate was prepared by mixing 7 ml fibrinogen solution (0.5% [w/v] human fibrinogen in phosphate buffered saline [PBS]; pH 7.4), 50 μl thrombin solution (100 NIH units/ml; Sigma), and 18 ml of 2% (w/v) agarose. The fibrin plate was left at room temperature for 1 hr to allow the formation of a fibrin clot layer. The filtered supernatant from *bacilli* cultivated for 40 hr or purified enzyme (0.3 μg) was spotted onto the plate and incubated at 37°C for 16 hr. Fibrinolytic activity was determined by measuring the diameters of the clear zone and was expressed in plasmin units (U) by comparing it to the zones formed by known quantities of plasmin. A standard curve, which showed the relationship between the area of the clear zone formed and

Table 1. Synthetic oligonucleotides used for site-directed mutagenesis

	Oligonucleotide sequences	
Wild type	aph-F: 5′-GAGCGATTGAAGCTTTGTACAAATACTC-3′	*Hind*III site
	aph-R: 5′-TCTTCAGAGGGATCCACCCGTCGA-3′	*Bam*HI site
Mutant		
AprE51-7	ap166R-F: 5′-GCTCAAGCACAGTGCGCTACCC-3′	mutated site
	ap166R-R: 5′-GGGTAGCGCACTGTGCTTGAGC-3′	mutated site
AprE51-8	ap218S-F: 5′-TACGGTGCGTACAGTGGTACGTC-3′	mutated site
	ap218S-R: 5′-GACGTACCACTGTACGCACCGTA-3′	mutated site

Underlined sequences denote the sites indicated

the number of plasmin units was prepared over a range of 2-60 mU. The activation of plasminogen by AprE51 variants was measured the dimension of the clear zone by comparing it to the zone formed by AprE51 variants with plasminogen (from human plasma, Sigma-Aldrich, St. Louis, MO) [14].

The optimal pH for the fibrinolytic activity of the enzyme was determined using *N*-Suc-Ala-Ala-Pro-Phe-*p*NA at 37°C within a pH range of 3.0-12.0 as previously described [13]. The effect of temperature on enzyme activity was tested by heating samples in 50 mM Tris-HCl (pH 8.0) from 25°C to 55°C for 5 min. The thermostability of AprE51-7 and AprE51-8 was determined by incubating the enzyme solution (AprE51-7, 2.1 nM; AprE51-8, 2.0 nM) in 50 mM Tris-HCl buffer (pH 8.0) at different temperatures ranging from 45 to 60°C.

Kinetic determination

Kinetic parameters were measured using the chromogenic substrate *N*-Succ-Ala-Ala-Pro-Phe-*p*NA. Samples (0.3 ml) from the reaction mixture containing the enzyme (AprE51-7, 0.84 nM; AprE51-8, 0.74 nM) and substrate (0.1-1.0 mM) in 50 mM Tris-HCl buffer (pH 8.0) at 37°C were taken at various time points over the course of the reaction. The reaction was stopped by addition 100 µl of ice-cold 0.2 M acetic acid. The absorbance of released *p*NA was measured at 405 nm. The kinetic constants were determined using the Michaelis-Menten equation on the basis of the initial reaction rates.

Degradation of the artificial blood clots

The thrombolytic effect of AprE51 variants was observed by artificial blood clot degradation. Fresh mouse (4 week-old male BALB/c) blood was collected by retro-orbital puncture with capillary tubes (Fisher Scientific). An artificial blood clot was made by spontaneous coagulation in an eppendorf tube using 1 ml of fresh mouse blood. After 3 hr the artificial blood clot was rinsed out with PBS buffer (pH 7.4) repeatedly [16]. The artificial blood clot was dipped into 3 ml of PBS (pH 7.4) containing the purified enzyme (25 µg) at 37°C for 2 hr. Normal saline was used as the positive control. After incubation, the residual blood clot was isolated and weighted. The dissolution ratio of thrombus was calculated using the following equation: the dissolution ratio of thrombus (%)=$(W_0-W_1)/W_0\times100$, where W_0 was the thrombotic weight formed in an eppendorf tube and W_1 was the residual thrombotic weight [24].

Results

Expression and purification of AprE51-7 and AprE51-8 mutants

Previous study revealed that AprE51-6 (triple mutant of AprE51, S101W/G169A/V192A) constructed by error-prone PCR and site-specific mutagenesis showed the strongest fibrinolytic activity among any other AprE51 mutants [13]. Earlier mutagenesis study of *B. subtilis* subtilisin E indicated that the residues of Gly-166 and Asn-218 are involved in substrate binding site, which explains their effects on catalytic activity and thermostability [6, 27]. Gly-166 and Asn-218 were targeted to change the amino acid residues in AprE51. The comparison of the amino acid sequence of AprE51 with *B. subtilis* subtilisn E showed that 166th and 218th amino acid residues were identical to glycine and asparagine in AprE51, respectively [12, 27]. Based on the mutagenesis study of *B. subtilis* subtilisin E, Gly-166 was mutated into arginine by site-directed mutagenesis and the resultant mutant was designated as AprE51-7. Asn-218 also changed into serine from AprE51-7 and the resultant mutant was designated as AprE51-8.

To express each AprE51-7 and AprE51-8 mutant, the expression plasmids, pHY51-7 and pHY51-8, were constructed and transformed into *B. subtilis* ISW1214 using electroporation method, respectively. After *Bacillus* transformants for overexpression of the AprE51 mutants were grown, the fibrinolytic activities were detected in the culture supernatant of the two transformants using *N*-Succ-Ala-Ala-Pro-Phe-*p*NA as a substrate. The enzymes secreted by both mutants were active. AprE51-7 and AprE51-8 were purified to electrophoretic homogeneity using ammonium sulfate precipitation, HiTrap Q HP ion exchange, and HiTrap phenyl HP hydrophobic interaction chromatographies. The purified enzymes showed the same size as a 27 kDa wild-type AprE51.

Fibrinolytic activities of AprE51 variants

The fibrinolytic activities of AprE51-7 and AprE51-8 were determined by measuring the areas of the lysed zone on the fibrin plate. AprE51-7 and AprE51-8 exhibited approximately 1.8- and 4.5-fold increase in the fibrinolytic activity compared to the wild-type enzyme AprE51, respectively, but showed smaller clear zone than AprE51-6 (Fig. 1 and Table 2). The kinetic parameters of the purified AprE51-7 and AprE51-8 were determined based on the intercepts of the

Table 2. Fibrinolytic activity of purified AprE51 mutants

Enzyme	Amino acid substitutions	Fibrinolytic activity (units/mg)	Fold	Reference
AprE51 (wild type)		310.3	1.0	[13]
AprE51-1	G169A	419.2	1.4	[13]
AprE51-4	S101W/G169A	1182.2	3.9	[13]
AprE51-6	S101W/G169A/V192A	1618.5	5.0	[13]
AprE51-7	S101W/G166R/G169A/V192A	556.7	1.8	this study
AprE51-8	S101W/G166R/G169A/V192A/N218S	1403.2	4.5	this study

Fibrinolytic activity was determined by the fibrin plate method by using plasmin as a standard, as described in the materials and methods section. Assays were done at 37°C for 16 hr at pH 7.4. Fold activity was calculated as the ratio of the fibrinolytic activity of each mutant to that of the wild-type enzyme.

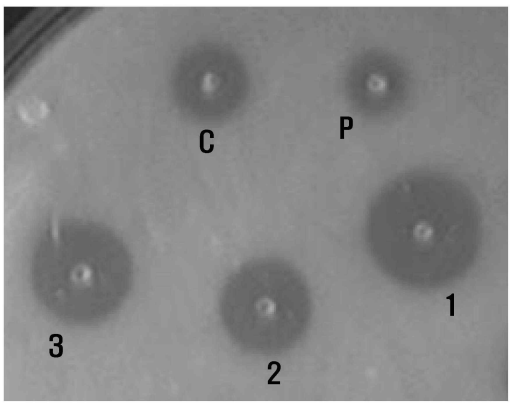

Fig. 1. Fibrinolytic activity of the purified AprE51 mutants in a fibrin plate. C, wild-type AprE51 as a control; P, 1 mU plasmin; 1, AprE51-6; 2, AprE51-7; 3, AprE51-8. The resulting data are shown in Table 2.

Michaelis-Menten equation based on the initial reaction rates. As shown in Table 3, the k_{cat} of AprE51-7 was 1.9 times higher than that of wild-type AprE51 but its K_m was 2.1 times lower, which resulted in an obvious increase (~3.8-fold) in catalytic efficiency (k_{cat}/K_m) compared to wild-type enzyme. The k_{cat}/K_m ratio of AprE51-8 exceeded that of the wild-type AprE51 by a factor of 4.7. An increase in k_{cat}/K_m is also affected by both k_{cat} and K_m values.

The optimum pH and temperature of AprE51 variants are shown in Fig. 2. All AprE51 variants showed similar pH profiles, which were active at neutral and alkaline pH values. AprE51-7 and AprE51-8 had relatively broader pH optimum than the wild-type enzyme AprE51. In addition, AprE51-7 and AprE51-8 maintained approximately 91% and 94% residual activities at pH 6.0, but AprE51-6 and AprE51 were 75% and 53%, respectively. The optimal temperatures of AprE51-7 and AprE51-8 were 54°C but those of AprE51 and AprE51-6 were 42°C. Obviously, a remarkable activity was observed in AprE51-7 and AprE51-8 at high temperatures up to 55°C we tested.

The effect of plasminogen on fibrinolytic activity of AprE51-8 was tested from fibrin plate analysis (Fig. 3). When the purified AprE51-8 (0.3 μg) with plasminogen (0-20 mU) were spotted on the plasminogen-free plate, fibrinolytic activities of AprE51-8 had no remarkable difference. This indicates that AprE51-8 is a direct degrader of fibrin, and has no ability of converting plasminogen to plasmin.

Thermostability of AprE51-7 and AprE51-8 mutants

Thermostability of AprE51-7 and AprE51-8 was measured by the rates of thermal inactivation (or half-life) at elevated temperatures [27]. The half-lives were determined by measuring the residual catalytic activity after incubation of the

Table 3. Kinetic constants of AprE51-7, AprE51-8, and nattokinase

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
AprE51[1]	0.35±0.01	231.3±18.4	6.61×10^5
AprE51-6[1]	0.15±0.03	586.2±96.7	3.90×10^6
AprE51-7	0.17±0.01	440.5±2.8	2.52×10^6
AprE51-8	0.18±0.10	571.0±87.2	3.10×10^6
Nattokinase[2]	0.31±0.01	45.2±3.2	1.43×10^5

Kinetic constants were determined using *N*-Succ-Ala-Ala-Pro-Phe-*p*NA as substrate in 50mM Tris-HCl buffer, pH8.0 at 37°C. Data was expressed as mean values of three replications with their corresponding standard deviations.

[1]Ref.13, [2]Ref.1.

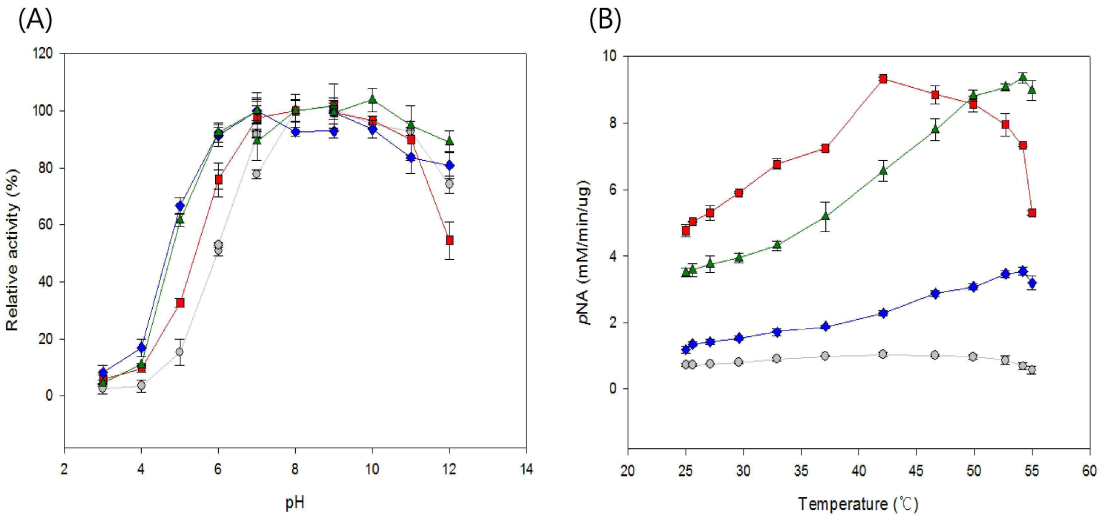

Fig. 2. Effect of pH and temperature on the activity of AprE51 (●), AprE51-6 (■), AprE51-7 (◆) and AprE51-8 (▲) mutants. The fibrinolytic activity of AprE51 variants were determined with *n*-Succ-Ala-Ala-Pro-Phe-*p*NA for 5 min at 37°C using various 50 mM buffers (citrate, pH 3.0-5.0; sodium phosphate, pH 6.0-7.0; Tris-HCl, pH 8.0-9.0; Glycine-NaOH, pH 10.0-12.0).

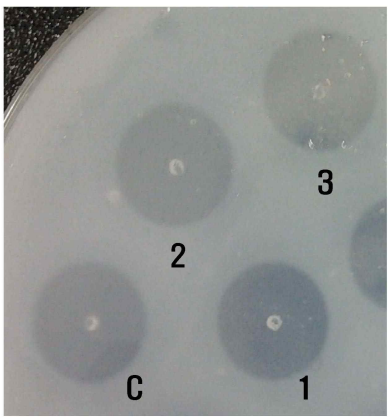

Fig. 3. The activation of plasminogen by AprE51-8. The purified enzymes (0.3 μg) with plasminogen were dropped onto the hole instead of enzyme alone. After incubation of the plate at 37°C for 16 hr, the activation of plasminogen was determined by measuring the dimension of the clear zone. C, AprE51-8 without plasminogen; 1, AprE51-8 with 2.5 mU plasminogen; 2, AprE51-8 with 5 mU plasminogen; 3, AprE51-8 with 20 mU plasminogen.

enzyme at indicated temperature for various time periods. The results of thermostability examination were shown in Fig. 4. After 60 min incubation period, AprE51, AprE51-6, AprE51-7, and AprE51-8 were stable at 45°C, but after 10 min at 60°C, the fibrinolytic activities of AprE51, AprE51-6, and AprE51-7 were completely lost. In case of AprE51-8, the activity was not completely lost. The half-lives of AprE51-7 and AprE51-8 at 50°C are 21.5 and 27.3 min, which are 2.0 and 2.6 times longer than that of the wild-type AprE51, respectively (Table 4). The rate of thermal inactivation of nattokinase at 55°C was found to be 7.8 min, and the half-lives of AprE51 mutants (AprE51-6, AprE51-7, and AprE51-8) and nattokinase at 55°C were similar [13, 23].

Blood clot degradation with AprE51-8 mutant

The *in vitro* fibrinolytic effects of the AprE51-6 and AprE51-8 were examined by an artificial blood clot degradation assay [14]. The blood clot degradation was observed in the test tube at 37°C by spreading the red blood cells, which were trapped by multiple fibrins net, in the solution after 2 hr (Fig. 5). After incubation, the degraded blood clot was isolated by removing the supernatant, and weighted. The dissolution ratios of thrombus in AprE51-6 and AprE51-8 solution (25 μg) were 18% and 25%, respectively (Table 5).

Discussion

In recent years, fibrinolytic enzymes such as nattokinase, CK11-4, and subtilisin DFE were isolated from Asian traditional fermented food and their physiochemical properties have been characterized [14, 17, 20]. Especially, to achieve higher stability of fibrinolytic enzyme against conditions of pH, temperature, or oxidation, many researchers have studied the directed evolution and capsulation. Recently, *B. amyloliquefaciens* CH51 strain was previously isolated from cheonggukjang [12]. AprE51, which was purified from this

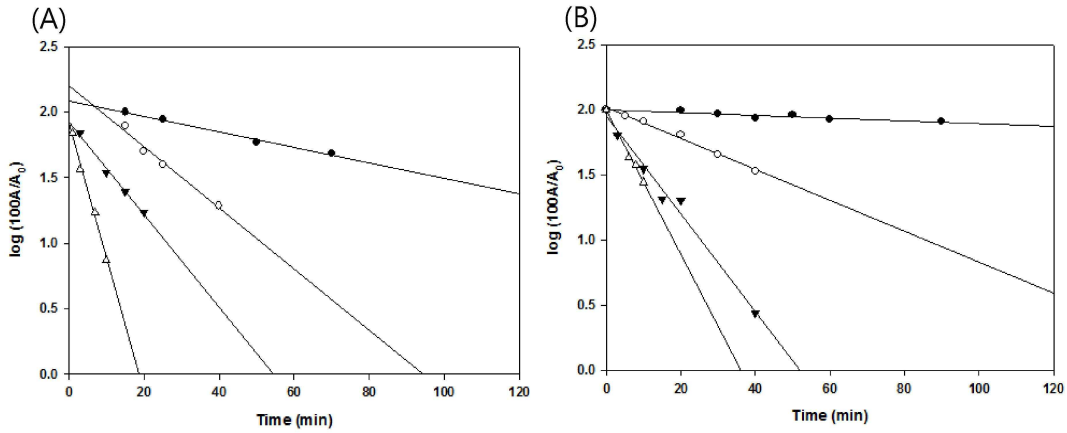

Fig. 4. Thermostability of (A) AprE51-7 and (B) AprE51-8 mutant. Thermostability of the enzyme was determined after incubation in 50 mM Tris-HCl buffer (pH 8.0) at 45°C (●), 50°C (○), 55°C (▼), and 60°C (△) for different incubation times. Residual activity was measured at 37°C in 50 mM Tris-HCl (pH 8.0) with *N*-Suc-Ala- Ala-Pro-Phe-*p*NA.

Table 4. Thermostabilities of the wild-type AprE51 and other AprE51 mutants

Enzyme	Half-life (min)			
	45°C	50°C	55°C	60°C
AprE51	60.4	10.6	3.3	-
AprE51-6	67.8	22.0	6.9	2.3
AprE51-7	68.3	21.5	6.4	2.3
AprE51-8	71.9	27.3	7.3	5.2

The fibrinolytic activities of the enzymes were determined at various temperatures by using *N*-Succ-Ala-Ala-Pro-Phe-*p*NA. After various time intervals, samples were withdrawn and the residual activity was measured at 37°C in 50mM Tris-HCl buffer (pH 8.0).

Table 5. The degradation of thrombus by AprE51-7 and AprE51-8 mutant

Enzyme	Weight of artificial blood clot (g)		Dissolution ratio (%)
	0 hr	2 hr	
Control (no enzyme)	0.330	0.330	0
AprE51-6	0.365±0.007	0.300±0.014	17.8
AprE51-8	0.280±0.008	0.210±0.009	25.0

The dissolution ratio of thrombus was calculated using the following equation: The dissolution ratio of thrombus (%) = $(W_o-W_1)/W_o \times 100$, where W_o was the artificial thrombotic weight formed as mentioned in Materials and methods and W_1 was the residual thrombotic weight after 2 hr incubation with enzyme.

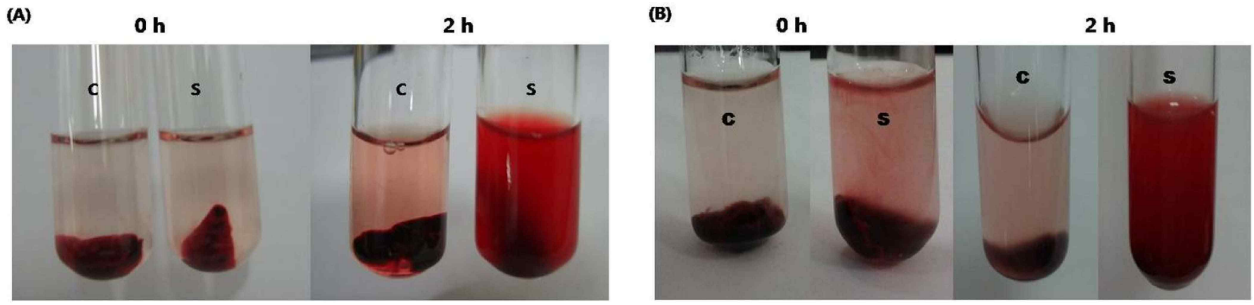

Fig. 5. Lysis of the artificial blood clots by (A) AprE51-6 and (B) AprE51-8. The artificial blood clots, formed by spontaneous coagulation using fresh mouse blood, were dipped in 3 ml PBS containing 25 μg purified AprE51 at 37°C. C, artificial blood clots with saline solution as a control; S, artificial blood clots with 25 μg of the AprE51-6 or AprE51-8.

strain, was a serine protease, and it showed relatively high fibrinolytic activity compared to nattokinase [13]. The gene encoding AprE51 was cloned and the deduced amino acid sequence was determined [12]. We previously succeeded to

obtain the mutant of AprE51, AprE51-6, which showed stronger fibrinolytic activity than the wild-type enzyme [15]. In this study, we attempted to improve the thermostability of the industrially potential candidate AprE51-6.

Previous attempts to study directed evolution to convert subtilisin E into a functional equivalent to thermitase revealed that the residues of Gly-166 and Asn-218 were involved in the substrate binding site, and the mutations of both residues affected the catalytic activity and thermostability of the enzyme [26, 27]. Therefore, AprE51-7 and AprE51-8 mutants were constructed by mutations of Gly-166 and Asn-218 residues. Each transformant was expressed as an active form. AprE51-7 and AprE51-8 enhanced the fibrinolytic activity of AprE51 by 1.8- and 4.5-fold in the fibrin plate assay, respectively, but had slightly lower fibrinolytic activity than AprE51-6. Especially, the substituted arginine in Gly-166 position of AprE51-7 is located on the bottom end of S1 pocket, which has been shown to be important in substrate binding [3, 22, 27]. Although the substitution of glycine for arginine reduces the accessible volume of the substrate binding site, it may lead to the decrease in the fibrinolytic activity because of the reduction in the hydrophobicity; thus, it may slightly affect the lower enzyme-substrate interaction and the ability to transform into product. This hypothesis is supported by the 1.3-fold lower k_{cat} value of AprE51-7 as compared to that of AprE51-6 (Table 3). The location of Asn-218 is involved in the binding of the C-terminal of leaving portion (P1´-P2´) of substrate [27]. The substituted serine of AprE51-8 may be attributed to enhance the turnover rate by the improvement of hydrogen bonding near substrate binding site. A slight increase of k_{cat} of AprE51-8 over AprE51-7 while its K_m remain unchanged supports this hypothesis.

AprE51-7 and AprE51-8 showed broad pH optima around alkaline conditions and their optimized pH values were very similar to nattokinase [21]. Above 80% of AprE51-7 and AprE51-8 activities were retained at pH values ranging from 6 to 12 whereas nattokinase maintained approximately 65% residual activities at pH 12 [8]. Thus, AprE51-7 and AprE51-8 would be more active than nattokinase at alkaline pHs. The optimum temperatures of the wild-type enzyme AprE51 and AprE51-6 were close to 42°C, but the substitution of Gly-166 located in S1 site with arginine increased the optimal temperature by 54°C (Fig. 2). The mutation of S1 site may contribute to increase the stability of the enzyme structure [18]. In case of AprE51-8, the optimum temperature was 54°C and the rate of thermal inactivation was slightly increased compared to AprE51-6. The substitution of Asn-218 with serine presumably increases the thermostability by slight improvement of hydrogen bonding network near a β-bulge, which is localized disruption of the regular hydrogen bonding of a β-sheet [27]. Therefore, AprE51-8 was more stable than AprE51 and AprE51-6, and may be better suited for industrial and pharmaceutical processes.

Previously, the comparison of fibrinolytic activity of the AprE51 mutants with other fibrionolytic enzymes such as subtilisin DFE, CK 11-4 from cheonggukjang, subtilisin BPN´, and subtilisin Carlsberg demonstrated that AprE51 mutant showed relatively high specificity for fibrin [13]. Although AprE51-8 successfully enhanced the thermostability of AprE51 and AprE51-6, the fibrinolytic activity may decrease in stomach due to its acidic condition when it is given orally as a thrombolytic agent. It is likely that most of the enzyme activity is lost via oral administration to the human gastric intestinal tract. Therefore, the pharmacokinetic study of this agent *in vivo* and the encapsulation methodology of enzyme would be further investigated for the clinical application.

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초록 : *Bacillus amyloliquefaciens* CH51이 생산하는 혈전용해효소의 열안정성 개선

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Bacillus amyloliquefaciens CH51은 분자량 27 kDa 크기의 subtilisin 타입의 혈전용해능을 지니는 단백질분해효소인 AprE51을 생산하였다. 이전연구에서 더 우수한 혈전용해 활성을 갖는 AprE51-6이 세포외 돌연변이법으로 생산되었으며, 본 연구에서는 이 개선된 효소인 AprE51-6의 열안정성을 증진시킬 목적으로 *B. subtilis* subtilisin E의 아미노산과의 상동성 분석을 통하여 두 아미노산인 Gly-166과 Asn-218이 치환되었다. 그 결과 G166R과 N218S 돌연변이체는 혈전용해능을 보이는 용해능 배지에서 원 효소보다 각각 1.8배와 4.5배 높은 혈전용해능을 보였다. 정제된 두 돌연변이효소인 AprE51-7과 AprE51-8는 원효소인 AprE51-6에 비하여 1.9 그리고 2.5배 높은 k_{cat}값을 나타내었고, 2.1과 1.9배 낮은 기질친화력을 나타내는 K_m값을 보여주었다. 특히 AprE51-8는 나토키나아제에 비하여 알칼리 pH 영역에서 높은활성을 유지하였고, 60°C에서 더 우수한 열안정성을 보여주었다. 열안정성의 정도를 나타내는 척도인 반감기 값에서도 AprE51-7과 AprE51-8는 50°C에서 21.5분과 27.3분으로 기존의 AprE51보다 2배 그리고 2.6배 더 긴 반감기를 보였다.